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PRINCIPAL INVESTIGATOR: Robert Dickson, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University Medical Center
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**6. AUTHOR(S)**
Robert Dickson, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Georgetown University Medical Center
Washington, DC 20057

**E-MAIL:**
dicksonr@gunet.georgetown.edu

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Although the antihormone tamoxifen blocks the early growth of breast cancer, in many cases even small, early tumors begin to invade a woman's body and are very difficult to cure. The answer to these problems may lay in the development of an anti-cancer dietary supplement. One of the most promising anti-cancer dietary components is the soybean and soy-derived products. Indeed, one of these products, called the Bowman-Birk Inhibitor concentrate (BBIC) has recently been approved for cancer prevention trials in people. Interestingly, the active ingredient of BBIC is a small protein that prevents the actions of a cancer to invade the body of the individual. Thus, the mechanism of action of BBIC is completely different from that of tamoxifen. We have now completely cloned a BBI-inhibitable protease, termed matriptase, which is dramatically increased in human breast cancer, where it is found embedded in the cell membrane and exposed to the outside of the cancer cell. We have also found one natural, Kunitz-type inhibitor of matriptase, termed HAI-1. Our study will focus on the mechanisms of interaction of BBI, BBI-related inhibitors, and HAI-1 with matriptase in solution and on breast cancer cells in culture. These studies have direct application for breast cancer prevention.

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Robert B. [illegible]

PI - Signature Date

## Table of Contents

## Introduction:

Current, successful strategies for prevention of breast cancer focus on early detection of the disease and on anti-hormonal methods for diminishing its onset and slowing its early progression. Mammography, a low fat diet, only moderate consumption of alcohol, and regular exercise are beneficial; all may work to decrease exposure of the mammary gland to estrogen (1-4). The first clinical trial to use tamoxifen in prevention of breast cancer in high risk women was recently completed with remarkable success; breast cancer incidence was reduced by 45%. The rationale of this trial was to utilize this antiestrogen to inhibit proliferation and progression of early lesions (5,6). However, it is not clear that antihormonal strategies alone will be sufficient to prevent the disease because even early breast cancers may be estrogen receptor negative. Development of alternative approaches to induce mammary epithelial differentiation (similar to early pregnancy) and/or to directly prevent the acquisition of invasive ability by an early lesion may now be appropriate (6,7). However, prevention approaches may also appropriately focus on better understanding of how to suppress expression of the genes (or the activity of their protein products) involved in acquisition of malignant and metastatic behavior of a premalignant lesion (8,9).

Epidemiologic studies have strongly suggested that a diet containing high levels of serine protease inhibitors are associated with a low incidence in cancers of the breast, colon, prostate and head and neck (10). Specific studies on Japanese and Seventh Day Adventist populations have implicated soybean products in this effect, although soybeans may contain multiple, additional anticarcinogenic components (11-13). Soybeans have

markedly high levels of antichymotrypsin activity (14). This activity is largely comprised of one principle molecule: The Bowman-Birk Inhibitor (BBI). Many studies have demonstrated its anti-carcinogenic activity *in vivo* and *in vitro* (15-16).

Despite these promising observations of cancer preventing - activity of BBI and other serine protease inhibitors, a target protease(s) has not been definitively identified to date. Hydrolysis of the artificial substrate Boc-Val-Pro-Arg-MCA has been examined in multiple carcinogenesis systems and has been observed to be reproducibly elevated; BBI inhibits this activity, thus defining an assay for isolating and characterizing the enzyme(s) responsible (17). Using inhibitor-affinity chromatography, a candidate, BBI-directed, 43-45 kDa protease(s) has been partially isolated from tumor promoter-treated mouse and human fibroblasts. This enzyme is reported to have a cytosolic localization (18-20). Despite these localization data, independent studies using fluorescent microscopy have established that BBI initially binds to an apparently different cell-surface molecule(s) on fibroblasts and is internalized by a process resembling receptor-mediated endocytosis (21). More recently, a BBI-inhibitable, cytosolic, 43 kDa, pH 8-active serine protease has been detected in MCF-7 breast cancer cell (22). Thus, to date, although the BBI appears to initially encounter a membrane target on cells, a target protease with this localization has not been isolated nor purified from any cell type.

We have carried out our studies on an epithelial-derived, integral membrane, trypsin-like, serine protease (matriptase) to test the hypothesis that matriptase could serve as membrane target for naturally occurring serine protease inhibitors. Matriptase is a type 2, integral membrane, trypsin-like serine protease with two putative regulatory modules: two tandem repeats of a CUB (C1r/s, Uegf and Bone morphogenetic protein-1)

domain and four tandem repeats of an LDL receptor domain (23) (also see updated sequence in the GenBank/EBI Data Bank with accession number AF118224). The cognate inhibitor of matriptase is a type 1 integral membrane protein, containing two Kunitz domains, separated by an LDL receptor domain (24). The inhibitor was independently characterized by others (25), as an inhibitor (HAI-1) of hepatocyte growth factor activator. Understanding the inhibition and interaction of matriptase with its cognate inhibitor and other nature serine protease inhibitor could provide a new avenues for development of chemoprevention agents.

## Body:

During the Jul 99-Jun 00 period we have addressed Aim 1 and a portion of Aim 2 of the original proposal:

*Aim 1: We will first establish in detail the mechanism and kinetics in solution of inhibition of the 80-kDa protease by the BBI compared to the Kunitz domain inhibitor (KSPI) we have previously identified.*

As the first step toward approach Aim1, we have first purified the protease (now termed matriptase) using a combination of CM-Sepharose and immunoaffinity chromatography (Fig.1). To demonstrate that the KSPI inhibitor (now termed HAI-1) can inhibit the activity of matriptase, both HAI-1 and matriptase were purified from

human milk, and maintained in pH 2.4 to prevent their association. As the pH of the solution was raised to pH 8.0 and incubated at 37°C, the formation of the 95-kDa matriptase/HAI-1complex rapidly occurred (Fig. 2 A). Binding of HAI-1 to matriptase occurred, as indicated by the shift of matriptase from the 70-kDa, uncomplexed form to the 95-kDa matriptase/HAI-1 complex. Uncomplexed matriptase became undetectable by immunoblot after 30 and 60 min. of incubation (Fig. 2 A). Strong gelatinolytic activity was observed for the 70-kDa, uncomplexed matriptase in a gelatin zymogram (Fig. 2 B), in contrast to the trace amounts of gelatinolytic activity that were observed for the 95-kDa matriptase/HAI-1 complex. In addition, the rate of cleavage of a synthetic, fluorescent substrate by matriptase was decreased following complex formation (Fig. 2 C). These results provide direct evidence that HAI-1 is an inhibitor of matriptase, and that the interaction of these two molecules results in catalytic inhibition that is acid sensitive and reversible.

Using purified matriptase, we have measured the Km and Vmax of matriptase for a variety of protease substrate peptides. Table 1 shows that the most reactive peptide substrates for matriptase are N-t-Boc-Gln-Ala-Arg-AMC with the Km of 4.887 uM and N-t-BOC-Bz-Glu-Ala-Arg-AMC with the Km of 3.808 μM. No released fluorescence was detected from the substrates for chymotrypsin or elastase (Table 1, peptide substrates 7, 8, 9). Matriptase appears to prefer to bind to peptides containing small side chain amino acids, such as Ala and Gly, at P2 site (Table 1, peptide substrates 1-5). Peptides containing P2 Ala are better substrates for matriptase than peptides containing P2 Gly (compare peptides 1, 2 with peptides 3-5). The binding affinity of matriptase to the former is about 30-fold higher that to the latter. Interestingly, a change from Gln to Glu

at the P3 site significant reduces the Vmax (Table 1, comparing peptide 1 with 2) without causing significant change on Km. Using the most reactive substrate N-t-Boc-Gln-Ala-Arg-AMC, the Ki for HAI-1 and BBI have been estimated to be 1 nM and 470 nM, respectively.

The limited inhibition of matriptase by BBI (relative to HAI-1) has urged us to search for other naturally occurring serine protease inhibitors with BBI inhibitory loop. This work began, in collaboration with Dr. S. Wang (Georgetown University), with the computer modeling of the 3-D structure of the catalytic domain of matriptase and the interaction between matriptase and the two Kunitz domains of HAI-1, the endogenous inhibitor of matriptase (Fig. 3). Using this molecular computer modeling approach, we have characterized a panel of small molecule inhibitors of matriptase (Table 2) as well as a natural serine protease inhibitor from sunflower seed (Fig. 4). The sunflower seed trypsin inhibitor contains a BBI-related inhibitory loop and exhibits much more potent inhibition of matriptase with a Ki value of 0.92 nM. **Because of the success in using this molecular modeling to search for small molecule inhibitors and natural serine protease inhibitors of matriptase, we would like to continue this approach (in preference to our initially proposed combinatorial, phage display approach) to optimize our small molecule inhibitors and peptide inhibitors. Optimization of peptide inhibitors will be based on the structure of inhibitory loops of BBI and HAI-1. We now believe that this approach will be greatly preferable to our originally proposed approach of phage display of BBI loop-related sequences.**

*Aim 2: We will next examine whether the BBI compared to KSPI are capable of binding the membrane-bound 80-kDa protease and promoting its cellular internalization.*

In order to lay further groundwork for this Aim, we have established conditions whereby we can first activate matriptase in a controlled fasion, prior to determining its interaction with BBI, BBI-related inhibitors, HAI-1, or physiological relevant matriptase substrates. We have demonstrated that matriptase is synthesized as a single-chain zymogen and acquires its activity by cleavage at the canonical activation motif of the enzyme. The single chain form of matriptase lacks binding affinity to HAI-1; single chain matriptase and HAI-1 were detected mainly in their uncomplexed forms in T-47D cells. In contrast, the complexed matriptase isolated from human milk can be converted to two fragments under reduced conditions (Fig. 5). The matriptase cleavage site, generating the two fragments was also identified within the matriptase activation motif (R-VVGGTDAD). To further investigate the cellular binding and internalization of matriptase-inhibitor complexes, we have established a model of controllable activation of matriptase on the surface of epithelial cells. Activated matriptase is prerequisite for the binding and internalization of matriptase-bound protease inhibitors. Using anti-two-chain matriptase-specific mAbs (that were recently generated) and other anti-matriptase mAbs that react against both active and latent matriptase, we have begun to explore the activation of matriptase. In a non-transformed, normal human mammary epithelial-derived cells (HMEC) 184A1N4, we observed that matriptase activation may be regulated by serum in the culture medium in these cells; matriptase is expressed as a single-chain zymogen, following growth of cells for 2 days in medium supplemented

with 0.5% fetal bovine serum (FBS), the activated, two-chain form of matriptase was only detected at negligible levels (Fig. 6 A and B, time 0). In contrast, an increase in the level of the two-chain matriptase was detected when cells were re-fed with IMEM medium containing 0.5% fetal bovine serum. This increase in the expression of the two-chain form started to be detectable within 10 min of serum stimulation (Fig. 6 panel A) and was maintained for up to 7 hrs, at which time the levels of two-chain form was strongly decreased (Fig. 6 B). The concentration of serum, rather than the availability of matriptase, appears to be the limiting factor for the activation of matriptase, since the duration of matriptase activation appears to depend on the amount of serum added to the cells. Active matriptase was still detected 16 hr after addition of serum, when the culture medium was supplemented with 5% instead of 0.5% of fetal bovine serum (Fig. 6 C). These results suggest that serum contains a factor which can induce the activation of matriptase in these HMEC cultures, and that this factor is consumed or inactivated by these HMEC, resulting in transient induction of activation of matriptase. We will next proceed with study of BBI interaction with matriptase following its controlled activation on cell surfaces.

## Statement of work

**Year 1:** In the first year we will carry out all enzyme kinetics studies on the soluble protease (Aim 1) [**Completed**]. In addition, we will begin studies on inhibitor and protease internalization in breast cancer cells (Aim 2) **[Completed].**

**Year 2:** In the second year, we will carry out phage display studies to further optimize BBI and HAI inhibitory domains (Aim 1). [**We now request, as discussed above to replace phage display methodology with molecular modeling for these Aim 1 studies**]. In addition, we will carry out catalytic inhibition studies of gelatin degradation by breast cancer cells (Aim 2).

**Year 3:** In the final year, we will carry out all proliferation and differentiation studies with the protease inhibitors. These will include the Myc, Mad, and casein regulation studies of Aim 3. In addition, if effective, peptide based inhibitors generated in year 2 will be tested in comparison to BBI and HAI-1, themselves.

## Key research accomplishments:

We have shown that:

- Active form matriptase can be purified from human milk by a combination of CM-Sepharose, immunoaffinity chromatography, and preparative gel elelctrophoresis.
- Matriptase exhibits trypsin-like activity by cleavage of various synthetic substrates containg Arg or Lys as P1 sites. The most reactive peptide substrate is N-t-Boc-Gln-Ala-Arg-AMC with the Km of 4.887 uM.
- The Ki values for HAI-1 and BBI on matriptase were estimated to be 1 nM and 470 nM, respectively.
- A panel of small molecule inhibitors with Ki values in the uM range and a natural, BBI loop-related serine protease inhibitor of sunflower seed with sub-nM Ki were identified by molecular modeling.
- Matriptase was shown to be synthesized as a single-chain zymogen prior to its conversion to a two-chain active form. Only the two-chain matriptase can bind to HAI-1.
- A controllable model for serum-dependent activation of matriptase on living mammary epithelial cells has been established.

## Reportable outcomes:

### Abstracts:

1. Valdman T., Lin C.-Y., and Dickson R.B., The Bowman-Birk inhibitor inhibits matriptase in breast cancer cells, Proceedings of the Annual meeting of the AACR, Philadelphia, PA, 1999.

2. Lin C.-Y., Enyedy I.J., Wang S., and Dickson, R.B., Structural basis of the interaction between matriptase and its cognate Kunitz-type inhibitor. Proceedings of the Annual meeting of the America Chemical Society, New Orleans, 1999.

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4. Lee, S.-L., Dickson, R.B., and Lin, C.-Y. Biological functions of matriptase, a novel trypsin-like protease. FACS/The Salk Institute for biological studies. 16th Annual Meeting on Oncogenes and Tumor Suppressors: The Evolution of the Cancer Cell. 2000

5. Enyedy, I.J., Lee, S.-L., Lin, C.-Y., Dickson, R.B., and Wang, S.. Structure based design of inhibitors for matriptase. Proceedings of the Annual Meeting of the American Chemical Society, Washington DC, 2000.

## Paper:

1. Lee, S.-L., Dickson, R.B., and Lin, C.-Y. Activation of hepatocyte growth factor and urokinase type plasminogen activator by matriptase, an epithelial membrane serine protease. Manuscript submitted to J. Biol. Chem.

2. Benaud, C., Dickson, R.B., and Lin, C.-Y. Multiple regulatory mechanisms of the activity of matriptase on the surfaces of mammary epithelial cells. Manuscript submitted to J. Biol. Chem.

3. Enyedy, I.J., Lee, S.-L., Kuo, A.H., Lin, C.-Y., Dickson, R.B., and Wang, S. Discovery of small molecule inhibitors of matriptase through 3-D database screening. Manuscript in preparation for Medicinal Chemistry.

4. Long, Y.-Q., Lee, S.-L., Lin, C.-Y., Enyedy, I., Wang, S., Dickson, R.B., and Roller P.P. Synthesis and evaluation of the sunflower derived trypsin inhibitor as a potent inhibitor of the matrix-degrading protease, matriptase. Manuscript in preparation for Bioorganic & Medical Chemistry Letters

## Conclusions:

We have successfully purified active matriptase by a combination of CM-Sepharose, immunoaffinity chromatography, and preparative gel electrophoresis. Using

this purified, active matriptase, we obtained evidence that the enzyme is a trypsin-like protease, by cleaving various peptide substrates containing Arg or Lys as P1 sites. The most reactive peptide substrate is N-t-BOC-Gln-Ala-Arg-AMC with the Km of 4.887 uM. Using this substrate, we were able to obtain the Ki value of HAI- 1 and BBI. Although BBI is clearly able to inhibit matriptase, this soy-derived inhibitor appears not to be optimally potent for blockade of matriptase.

To further search for other natural, BBI-related, serine protease inhibitors which exhibit stronger inhibition of matriptase, and for other small molecule inhibitors of matriptase, we have obtained a 3-D model of the catalytic domain of matriptase and studied its interaction with each Kunitz domain of HAI-1. Using this methodology, we discovered a natural inhibitor from sunflower seed. This sunflower seed trypsin inhibitor exhibits strong inhibition of matriptase with a Ki below one nM and a high selectivity. This inhibitor will provide us a valuble agent to further study the role of matriptase in breast cancer progression and could have potential as a chemoprevention agent. Furthermore, using our 3-D molecular modeling, we are able to screen small molecule inhibitors of matriptase with reasonable potency and selectivity. These small molecule inhibitors will be used as a lead compounds to develop more selective and potent inhibitors for matriptase.

We have also established a controllable system to regulate activation status of matriptase on living epithelial cells. Using this system, we will investigate how serine protease inhibitor, such as HAI-1, BBI, and sunflower trypsin inhibitor regulate matriptase activity, and in turn affects the cellular activity, such as cell proliferation, degradation of extracellular matrix and cell migration.

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## Appendices:

**Table 1. Kinetic parameters of matriptase for various peptide substrates.**

Ten µl of matriptase solution and 10 µl of peptide substrate were added to a cuvette containing 180 µl reaction buffer, the release of fluorescence resulting from hydrolysis of the peptide was recorded in a fluorescent spectrophotometer at room temperature with excitation at 360 nm and emission at 480 nm. Km and Vmax were determined by double reciprocal plot derived from Michaelis-Menten equation.

| PeptideSubstrate | P4--P3--P2--P1-Amc | Km (uM) | Vmax (nM AMC/min) |
|---|---|---|---|
| 1 | Gln-Ala-Arg[a] | 4.887 | 654 |
| 2 | Glu-Ala-Arg | 3.808 | 76.3 |
| 3 | Leu-Gly-Arg[a] | 13.6 | 308 |
| 4 | Gln-Gly-Arg | 33.52 | 528 |
| 5 | Glu-Gly-Arg | 47.5 | 170 |
| 6 | Ala-Phe-Lys | 69.9 | 524 |
| 7 | Leu-Leu-Val-Tyr[a] | \\\\\\ [b] | \\\\\\ [b] |
| 8 | Ala-Ala-Pro-Phe[a] | \\\\\\ [b] | \\\\\\ [b] |
| 9 | Ala-Ala-Ala[a] | \\\\\\ [b] | \\\\\\ [b] |

[a] Gln-Ala-Arg is a standard substrate for trypsin, Leu-Gly-Arg is a substrate for uPA, Leu-Leu-Val-Tyr and Ala-Ala-Pro-Phe are the substrates for chymotrypsin, and Ala-Ala-Ala is the substrate for elastase.

[b] No cleavage activity was detected with these substrates at concentration of 200 µM.

**Figure 1: Purification of matriptase in its 95-kDa complexed form from human milk.**

The partially purified 95-kDa matriptase complex from ion exchange chromatography was loaded onto a mAb 21-9-Sepharose column. The bound proteins were eluted by glycine buffer, pH 2.4, and neutralized by addition of 2M Trizma base. The eluted proteins were incubated in 1 X SDS sample buffer in the absence of reducing agents at room temperature (lanes 1 each panel, boiling -) or 95°C (lanes 2 each panel, boiling +) treatment for 5 min. The samples were resolved by SDS-PAGE and either stained by colloidal Coomassie (panel A), or subjected to immunoblot analysis using mAb 21-9 (panel B), or subjected to gelatin zymography (panel C). The 95-kDa matriptase complex was eluted from this affinity column as the major protein (panel A, lane 1); it was recognized by mAb 21-9 (panel B, lane 1), and it also exhibited gelatinolytic activity (panel C, lane 1). The 95-kDa matriptase complex was converted to matriptase by boiling (panel A, lane 2). The gelatinolytic activity of the 95-kDa protease was destroyed by boiling, but a low level of the gelatinolytic activity was survived and converted to matriptase (panel C, lane 2). A low level of uncomplexed matriptase was co-purified with the 95-kDa matriptase complex by affinity chromatography (panel A, lane 1); it also exhibited gelatinolytic activity (panel C, lane 1). Immunoblot analysis enhanced the signal of the uncomplexed matriptase and reconfirmed its existence (panel B, lane 1). Several other polypeptides were also seen (panel A, lanes 1 and 2). Some of them could be the degraded products of the protease, since they were recognized by mAb 21-9 after longer exposure to the x-ray film. A 40-kDa protein doublet was seen in low levels in a non-boiled sample (panel A, lane 1), but its levels were increased after boiling (panel A, lane 2). This 40-kDa doublet was not recognized by mAb 21-9 (panel B). We propose that these two polypeptides could be binding proteins of matriptase. In the figure, MW stands for the molecular weight markers; their sizes are as indicated.

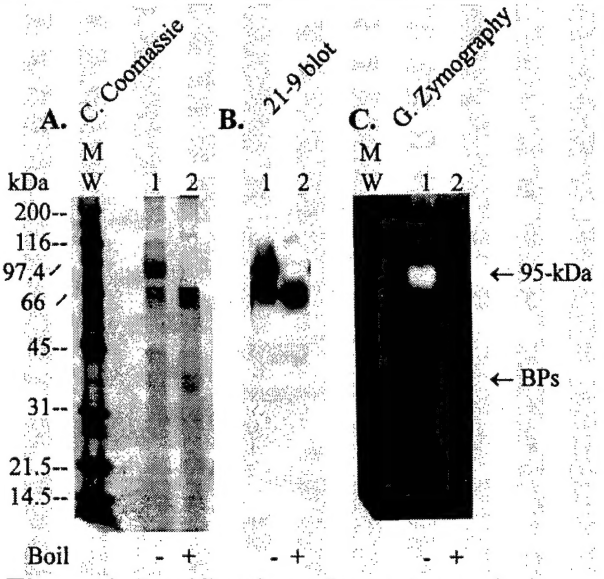
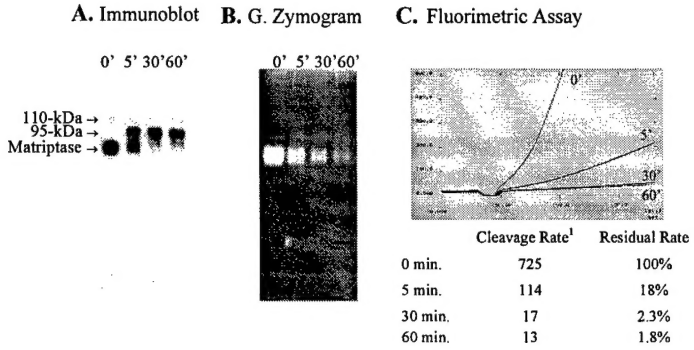



**Figure 1: Purification of matriptase in its 95-kDa complexed form from human milk.**

**Figure 2. Interaction with and inhibition of matriptase by HAI-1.**

Matriptase and HAI-1 were isolated from human milk by anti-matriptase mAb 21-9 immunoaffinity chromatography, as described previously, and were maintained in an uncomplexed status in elution buffer, 0.1 M glycine, pH 2.4. This preparation was brought to pH 8.5, incubated at 37°C for 0, 5, 30, and 60 min, and subjected to immunoblot using anti-matriptase mAb 21-9 (panel A), to gelatin zymography (panel B), and to a cleavage rate assay using the synthetic, fluorescent substrate, BOC-Gln-Ala-Agr- 7-amido 4-methylcoumarin (panel C). At zero min, matriptase was detected in its uncomplexed form (panel A), exhibiting strong gelatinolytic activity (panel B), and cleavage of soluble substrate at rapid rate (panel C). After 5 min incubation at 37°C, matriptase was detected both in an uncomplexed and complexed forms (panel A); the uncomplexed matriptase exhibited gelatinolytic activity, while much weaker activity was observed for complexed matriptase (panel B); the cleavage rate for fluorescent substrate was significantly reduced, down to 18% (panel C). After 30 and 60 min incubation, matriptase was detected mainly in an complexed form (panel A); negligible activity was observed by gelatin zymography (panel B) and by cleavage of fluorescent substrate. Also indicated, for comparison, is a milk-derived, matriptase-related 110-kDa protease (as indicated in panel A), which was not a complex of matriptase and HAI-1, and whose migration on SDS gel was reduced after boiling.




| | Cleavage Rate[1] | Residual Rate |
|---|---|---|
| 0 min. | 725 | 100% |
| 5 min. | 114 | 18% |
| 30 min. | 17 | 2.3% |
| 60 min. | 13 | 1.8% |

**Figure 2. Interaction with and inhibition of matriptase by HAI-1.**

**Figure 3. The interaction between the serine protease domain of matriptase and the Kunitz domains of HAI-1, as deduced by computer modeling.**

The interaction between the serine protease domain (green), the Kunitz domain 1 (Left panel), and the Kunitz domain 2 (Right panel) of HAI-1 (red) were deduced by computer modeling. The structures obtained from homology modeling were refined, using the molecular dynamics program CHARMM, with the all atom parameter set CHARMM22. Next, the Kunitz domains were placed, with the last massive atom of the P1 residue, Arg-260 (in Kunitz domain 1) and Lys-385 (in Kunitz domain 2), at 17-19 Å from Asp-185 carboxyl carbon and facing the S1 site. Self-guided molecular dynamic simulation (SGMD) was used to dock each Kunitz domain into the active site of matriptase. A time step of 1fs and a temperature of 300 K were used in all simulations. The Hoenig solvation model was used to represent the solvation effects. A restraining potential was used for gradually decreasing the distance between the P1 residue of HAI-1 and the S1 site of matriptase. The final distance between the two residues was set to be between 2.2 and 6.0 Å. Matriptase was fixed for the first 100-280 ps to save computer time. This was followed by 100 ps SGMD, without constraining matriptase.

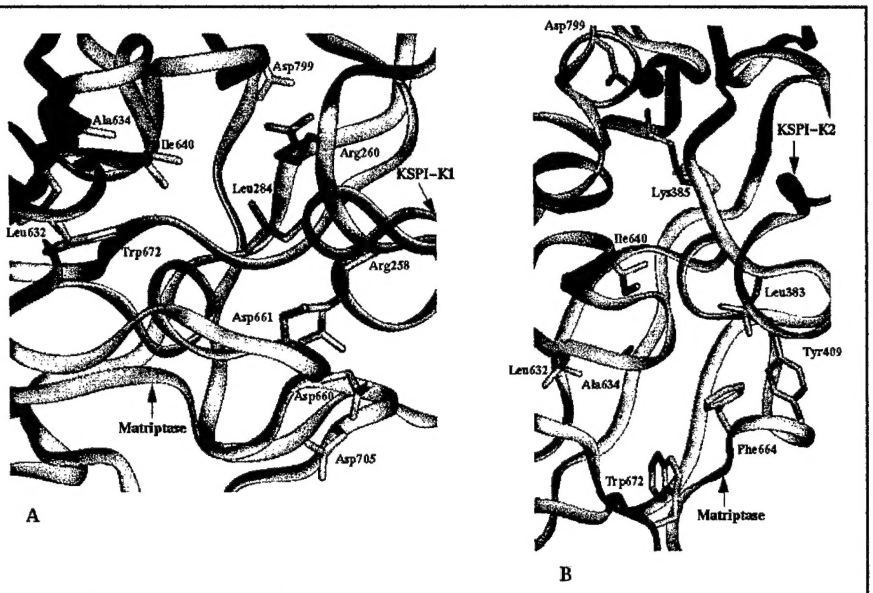



**Figure 3. The interaction between the serine protease domain of matriptase and the Kunitz domains of HAI-1, as deduced by computer modeling.**

**Figure 4: The structure of the complex of matriptase with SFTI-1 obtained after docking using molecular dynamics with the generalized effective potential.** The sunflower-derived SFTI-1 displayed a Ki of 0.92nM for matriptase

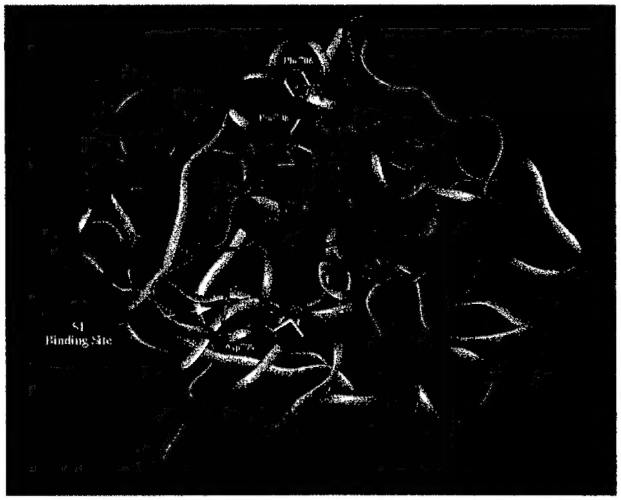



**Figure 4: The structure of the complex of matriptase with SFTI-1 obtained after docking using molecular dynamics with the generalized effective potential.**

**Figure 5. Non-reduced/reduced diagonal gel electrophoresis of complexed and uncomplexed matriptases.**

Matriptase was purified by immunoaffinity chromatography using anti-matriptase mAb 21-9 from conditioned medium of T-47D cells and from human milk. These samples were treated with SDS sample buffer in the absence reducing agents, incubated at 95°C for 5 min, and then resolved by SDS-PAGE (1st-D boiled). In the preparation from T-47D cells, only the uncomplexed form was purified, as there was no HAI-1 that was co-purified (panel A, 1st-D). In the matriptase preparation from human milk, the 95-kDa matriptase complex was converted to matriptase and the 40-kDa fragment doublet of HAI-1 under boiled, but non-reduced conditions (panel B 1st-D). A parallel gel strip was sliced, boiled in 1X SDS sample buffer in the presence of reducing agents for 5 min, placed on a second SDS gel, and electrophoresed. After these procedures, uncomplexed matriptase (panel A) remained as a single chain. Complexed matriptase (panel B), however, was dissociated into two components with apparent sizes of 45-kDa (A chain) and 25-kDa (B chain).

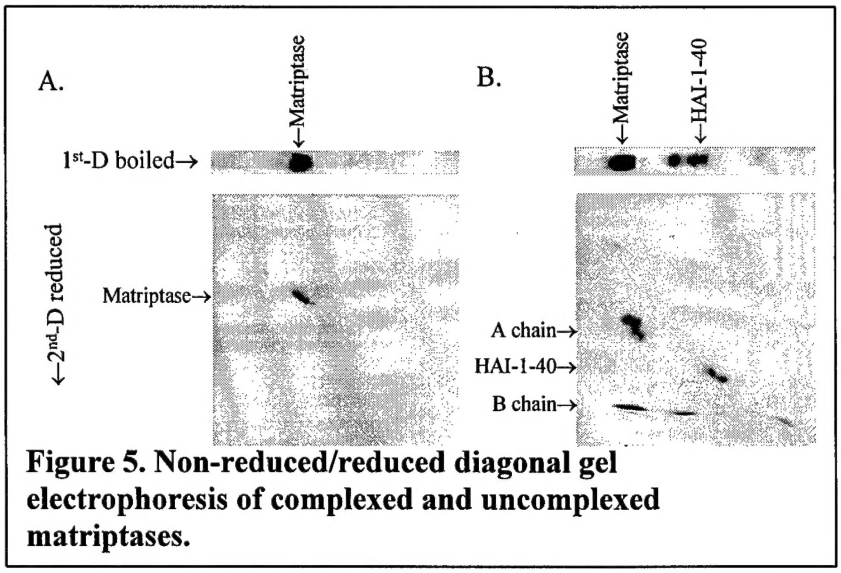



**Figure 5. Non-reduced/reduced diagonal gel electrophoresis of complexed and uncomplexed matriptases.**

**Figure 6: Transient activation of matriptase by serum.**

Non-transformed human mammary epithelial cells were cultured for 2 days in medium supplemented with 0.5% FBS. Cells were then exposed to fresh medium supplemented with 0.5 % FBS (Panels A and B) for the indicated times. The cells were harvested, and expression of two-chain matriptase, total matriptase, and matriptase inhibitor, HAI-1 were analyzed by immunoblot using mAbs, M69, M32, and M19, respectively. In panel C, low-serum starved cells were cultured with medium supplemented with either 0.5% or 5% FBS for 16 hrs, and expression of two-chain matriptase was determined.

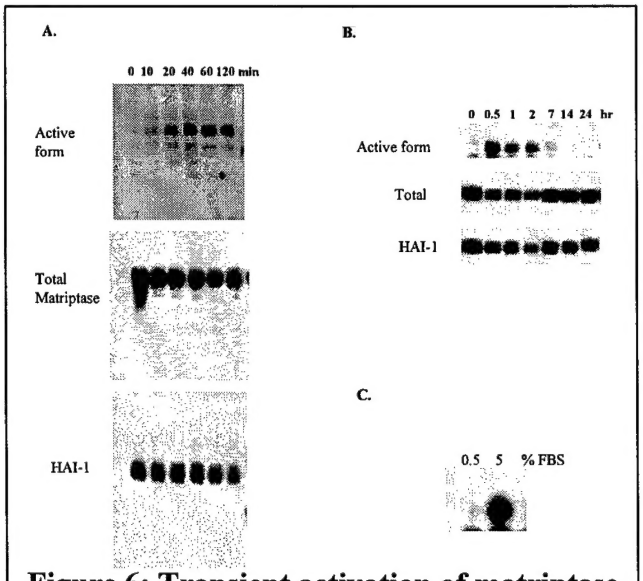



**Figure 6: Transient activation of matriptase by serum.**